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(21) International Application Number: PCT/CA91/00141 (22) International Filing Date: 26 April 1991 (26.04.91) (30) Priority data: 515,490 30 April 1990 (30.04.90) US (71) Applicant: THE UNIVERSITY OF BRITISH COLUMBIA [CA/CA]; Office of Research, Services and Industry Liaison, IRC 331-2194, Health Sciences Mall, Vancouver, British Columbia V6T 1W5 (CA). (72) Inventors: CHEUNG, Lori, Lan, Cho ; 3065 East Broadway, Vancouver, British Columbia V5M 1Z3 (CA). McNEIL, Bernard, Kelly ; 8169 Oak Street, Vancouver, British Columbia V6P 4A8 (CA). AUTOR, Anne, Pomeroy ; #9 - 766 West 7th Avenue, Vancouver, British Columbia V5Z 1B8 (CA). FERRIS, James, Alexander, Johnston ; 4149 Burkehill Road, West Vancouver, British Columbia V7V 3M6 (US).		(74) Agent: BEN-OLIEL, Susan, M., M.; McCarthy Tetrault, 1300-777 Dunsmuir Street, Vancouver, British Columbia V7Y 1K2 (CA). (81) Designated States: AU, CA, DE, GB. Published <i>With international search report.</i>
(54) Title: METHOD OF ESTABLISHING IDENTITY		
(57) Abstract A method for identifying an individual member of a species of organism comprises analyzing the DNA of the organism in respect to the presence or absence in the genome of one or more polymorphic enzyme sites and based on the comparison of these results to the presence or absence of one or more polymorphic enzyme sites in the DNA of one or more comparative members of the species, so characterizing the individual member of the species.		

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Method of Establishing IdentityTechnical Field

The present invention provides a biological method for identifying an individual member of a species of organism. This method is particularly useful in forensic science.

Background Art

There are many situations when the ability to determine accurately the identity of an individual member of a species of organism is of utmost importance. These include forensic applications such as the identification of missing persons, the identification of bodies after catastrophic accidents, the establishment of the presence of suspects at the scene of a crime and the matching of physical evidence left at the scene of a crime with a particular suspect. Other situations in which the establishment of accurate identification is crucial include family identifications, more specifically, maternal or paternal determination.

Current genetic analysis technology used for general individual identification and forensic identification relies upon the comparison of polymorphic regions in the DNA extracted from the individual who is to be identified and the frequency of those same regions in members of a general population to which the individual belongs. This "exclusion" technique becomes more accurate as the number of investigated polymorphic regions increases.

Canadian Patent No. 1,215,304 to Actagen Inc. describes a method of identifying an organism by analyzing the DNA of the organism with respect to one

SUBSTITUTE SHEET

-2-

or more polymorphic genetic regions and characterising the individual based on the differences in the polymorphisms in terms of relative size of the genetic regions. Specifically, this test is designed for paternity analysis and forensic testing. The DNA derived from the source to be analyzed is digested with one or more restriction enzymes and the resulting fragments are separated on the basis of size by causing them to migrate through a gel matrix under the influence of an electric current. The polymorphisms are detected by hybridizing the above-treated DNAs with labelled (e.g. radioactive) "probe" DNAs. The reacted probe DNAs allow visualization of the position and thus the size of the DNA fragment from the organism with which they are homologous. The size and position of the DNA fragment identified by its complementary DNA probe is variable among individuals in a given population of organisms as the probe DNAs are purposely chosen to be one allele from a polymorphic locus. Thus, if a probe is chosen correctly, it is polymorphic and not monomorphic, and DNA fragments of different sizes from different individuals will hybridize to the probe. If the probe is not chosen correctly, that is, it represents a monomorphic region, then differentiating between the individuals would be impossible.

In theory, the concept of using DNA polymorphisms as a genetic or molecular "fingerprint" is very good; however, the practice of this theory by way of the particular method described in Canadian Patent 1,215,304 is deficient in many aspects.

Firstly, the selection of the appropriate probe may be time-consuming. Without an appropriate

SUBSTITUTE SHEET

-3-

probe to a polymorphic genetic region, comparison to the general population and ensuing identification of the individual would not be possible. Secondly, this technique demands a generous biological sample from which to extract the DNA for testing. In many forensic cases, sample sizes, for example, at the scene of a crime, may be very limited and the genetic material that may be extracted therefrom may be partially or mostly degraded. Not only must there be a fairly large sample of undegraded DNA available for hybridizing with a selected probe, but it is indicated that there should be enough DNA to hybridize with up to 40 different probes. Thirdly, the number of alleles at each polymorphic genetic locus may be large and in fact may be as many as 50 to 100. It has become apparent that when dealing with this number of alleles, there may be considerable variability in the results. Fourthly, the sequential probing step may take one week per probe which translates into 4-5 weeks for 4-5 probes. This added to the time necessary for extraction to the filter results in a 6-week process. Although this may not be an issue in family identification testing, in forensic medicine time is of the essence, due to the pressure and necessity of expediently providing legal authorities with information.

Similar technology for the genetic identification of individuals relies upon the comparison of variable numbers of tandem repeats (VNTR) in polymorphic loci of DNA extracted from blood and other biological material that contains cells. Specifically, the DNA sample to be analyzed is digested by one or several restriction endonucleases and separated by fragment size using

SUBSTITUTE SHEET

-4-

gel electrophoresis. Using the Southern Blot technique, single stranded DNA on the gel is carried out of the gel onto a nylon membrane (filter) where it binds. The VNTR probes, which detect alleles ranging in sizes between approximately five hundred to eight thousand base pairs, are hybridized to the filter and thus the complementary strands of the cleaved single stranded DNA under investigation can be identified and visualized using autoradiography.

The VNTR regions are chosen as there is a continuum of allele sizes between different members of a given population, that is, not all individuals have the same number of tandem repeats. Based on the analysis of numerous VNTR loci, that is, the sizes of the alleles, individual identification can be made.

The problems with VNTR identification are as described above for the method of Canadian Patent No. 1,215,304. A major difficulty arises in the analysis of VNTR regions when only a small sample of material containing undegraded DNA is available for testing. In addition, the continuum of allele sizes in the VNTR regions, which can lead to differences between alleles as small as one base pair, may produce anomalies in the positioning of the bands separated by gel electrophoresis. Due to the nature of the electrophoretic technology, these minute differences in allele position are not easily resolved.

In addition, the technology involved in VNTR analysis is quite complex. This necessitates that the laboratory technicians charged with running the tests be well-educated in the steps of VNTR protocol and the concepts behind the procedure in

SUBSTITUTE SHEET

-5-

order to analyze meaningfully the gel results. Although the ideal situation, not all lab technicians are able to comprehend the background of the VNTR method, which in and of itself may lead to errors in the operation of the procedure and ultimately in the analysis of the results. Similarly, in forensic cases, as the VNTR laboratory results may be used as evidence in court proceedings, the jury and judges must be educated in the technology. The difficulty may be compounded when trying to explain anomalies in the gel results.

Aside from the obvious problems associated with the complexity of VNTR technology, other disadvantages include the time and labour cost involved in Southern Blotting, probe creations, hybridizations and the necessity of using radioisotope probes which not all laboratories are able to handle.

Accordingly, there is the need for a simple, accurate and rapid method of identifying an individual, which method obviates and mitigates the disadvantages outlined above and most importantly, which may be workable with even a small biological sample.

Disclosure of the Invention

The present invention provides a method for identifying an individual member of a species of organism which comprises analyzing the DNA of the organism in respect to the presence or absence in the genome of one or more polymorphic enzyme sites and based on the comparison of these results to the presence or absence of one or more polymorphic enzyme

SUBSTITUTE SHEET

-6-

sites in the DNA of one or more comparative members of the species, so characterizing the individual member of the species.

In other words, the analysis of the presence or absence of one or more restriction enzyme sites in the individual, coupled with the statistical analysis of the likelihood in the comparative member(s) of each particular restriction enzyme site being present or absent, will give excellent discrimination between individual members of a species of organism.

By way of background, each individual member of a species of organism, whether it be a member of an animal species (e.g. horses, cows, dogs, etc.) or a member of a mammalian species (e.g. humans...) possesses in its DNA, all of its genetic information. The DNA comprises a series of nucleotide base pairs arranged on each chromosome to form specific genes. The site on the chromosome where a gene is located is referred to as its locus. The human genome consists of 23 chromosome pairs, with one member of each pair being donated by each parent. Due to the contributions from each parent unit, in one individual there are two possible alternative forms (alleles) for each gene. Accordingly, the nucleotide base pair sequence on each chromosome may be different on each pair of chromosomes.

The present invention is based in part on simple genetics. Restriction enzymes or endonucleases cleave specific sites in a DNA chain. The enzymes bind to a certain number of nucleotide base pairs and only make cuts at specific sequences of nucleotides which are quite often palindromes.

SUBSTITUTE SHEET

-7-

With regard to each polymorphic restriction enzyme site, an individual, depending on the genetic information inherited from each of its parents, will be either homozygous for the presence of the restriction enzyme site, homozygous for the absence of the restriction enzyme site, or heterozygous.

If the individual possesses, on both chromosomes, the appropriate base pair sequence through which the restriction enzyme may cleave, then this individual is homozygous for the presence of the restriction enzyme site. However, if this individual possesses, on both chromosomes, base pair sequences at the loci or enzyme site which do not allow cleavage by the restriction enzyme, this individual is homozygous for the absence of the restriction enzyme site. Alternatively, if the individual possesses on one chromosome the appropriate base pair sequence for the cleavage by the restriction enzyme and does not possess this sequence at the enzyme site on the other chromosome, this individual is a heterozygote.

By analyzing the presence or absence of restriction enzyme sites at more than one loci in the individual and comparing the results to the statistics of the variability of the restriction enzyme site(s) in the comparative member(s), excellent individual identification by way of exclusion may be achieved.

Given that one of the greatest limitations of DNA technology, as it applies to the identification of individuals specially in forensic science, is the quantity and quality of DNA available for analysis, the present invention provides a

SUBSTITUTE SHEET

-8-

simple, workable solution. This method obviates the need for probes (radioactive or otherwise), hybridization, and Southern Blot technology and simplifies the interpretation of the results.

Other aspects of the invention will become evident upon reference to the following non-limiting drawings:

Brief Description of the Drawings

Figure I is a representation of the amplified APRT and PALB loci.

Figure II represents the amplified APRT locus from ten individuals as viewed on an electrophoretic gel.

Figure III represents the amplified APRT locus which has been digested and separated as bands on an electrophoretic gel.

Figure IV represents the amplified PALB locus from 12 individuals as viewed on an electrophoretic gel.

Figure V represents the amplified PALB locus which has been digested and separated as bands on an electrophoretic gel.

Figure VI represents the electrophoretic gel of the restriction digest control for the APRT locus.

Figure VII represents the simultaneous amplification of the APRT and PALB loci as viewed on an electrophoretic gel.

SUBSTITUTE SHEET

Best Mode for Carrying Out the Invention

Prior to setting forth the details of the invention, it may be helpful to an understanding thereof to provide definitions of certain terms:

alleles - alternative forms of a DNA sequence at a specific site on homologous chromosomes

polymorphic - a trait for which two or more alleles occur commonly in a population

restriction enzyme - enzymes that cleave DNA at specific base pair sequences, quite commonly at palindromes

polymorphic restriction enzyme site - refers to the DNA sequence that contains the restriction enzyme recognition site. This site may be present or absent in an individual in a population

primers - a DNA or RNA sequence that serves to initiate DNA synthesis

polymerase chain reaction - technique that allows for the enzymatic amplification of nucleic acid sequences using primers and inducing agents

gel electrophoresis - a process in which an electric field is used to move DNA fragments through porous gels.

Briefly stated, the present invention provides a simple and reliable method for identifying an individual member of a species of organism. As

SUBSTITUTE SHEET

-10-

described above, the DNA of the individual is analyzed with respect to the presence or absence in the genome of one or more polymorphic restriction enzyme sites and these results are compared to a similar analysis in one or more comparative members of the species.

In a preferred form, the presence or absence of the polymorphic restriction enzyme site(s) is determined by the following steps which comprise:

- (a) isolating DNA of the individual to be analyzed;
- (b) amplifying at least one specific nucleotide sequence of said isolated DNA which sequence comprises a polymorphic restriction enzyme site to produce amplified DNA;
- (c) digesting said amplified DNA with a restriction enzyme which is capable of cleaving the DNA at the polymorphic restriction enzyme site to produce DNA fragments;
- (d) separating the digested DNA fragments; and
- (e) identifying the presence or absence of cleavage at the restriction enzyme site.

In one embodiment of this invention, the comparative members are representative of the general population to which the individual member belongs.

SUBSTITUTE SHEET

-11-

In a second embodiment, the comparative members are selected from the group comprising the mother of the individual member, the father of the individual member and a sibling of the individual member. Accordingly, family identification, more specifically, maternal and paternal identification can be made.

In a third embodiment, the sample from the comparative member is being treated to determine if it is one and the same as the sample of the individual member. This is particularly important in establishing the presence of suspects at the scene of a crime and the matching of physical evidence left at the scene of a crime with a particular suspect.

The species to which said individual member belongs may be selected from the group comprising humans, animals, plants, viruses, bacteria, algae and fungi.

The DNA sample is obtained from the cells of adults, juvenile, fetal or embryonic tissue.

The genomic DNA may be isolated from the individual under consideration by standard procedures. The exact process in part, will depend on the nature of the cell sample from which the DNA is to be isolated, i.e. blood, tissue, semen, urine, or other cell-containing materials of the body; however, in general, the cells are lysed and the released DNA recovered through extraction such as a phenol/chloroform extraction with a final precipitation in, for example, alcohol.

SUBSTITUTE SHEET

-12-

Polymorphic restriction enzyme sites or loci must be identified in the genome of the species of organism to which the individual belongs. For example, if the individual is a human, the Human Genome Workshop Reports Cytogenetics and Cell Genetics can be scanned and polymorphic restriction enzyme sites easily identified. By surveying statistics of the particular chosen enzyme, it is possible to determine the frequency of the presence or absence of that restriction enzyme site in a general population of organism to which the individual is a member.

Ideally, and in a very preferred embodiment, the presence or absence of the chosen polymorphic restriction enzyme site should be as close to 50%/50% as possible. The more the ratio differs from this median, the greater the number of polymorphic restriction enzyme sites that may have to be identified and tested as described hereinbelow.

Once one or more polymorphic restriction enzyme sites have been chosen for analysis, the nucleotide sequence of each site, including the nucleotides flanking the restriction enzyme site must be ascertained. Again, in the case of human analysis, this information is available through the Human Genome Workshop Reports or through other published scientific literature. Particularly useful for this information are the computer data-bases including but not limited to, European Molecular Biology Laboratory (EMBL) and Genebank.

An important feature of the method of the present invention is that the nucleotide sequence of

SUBSTITUTE SHEET

-13-

the restriction enzyme site under consideration is amplified prior to examination of the effect of the restriction enzyme on that site. Accordingly, even though the sample size of undegraded DNA may be small, the amplification of the DNA sequences including the regions flanking the polymorphic restriction enzyme site allows for a more reliable statistical analysis of the results.

One appropriate method for amplifying DNA makes use of the polymerase chain reaction. It is to be understood, however, that other DNA amplifying techniques may fall within the scope of the present invention.

The amplification based on the polymerase chain reaction, of at least one specific nucleotide sequence of the "isolated" DNA from the individual, which sequence comprises a polymorphic restriction enzyme site, makes use of primers and, inducing agents, sometimes referred to as enzyme catalysts. This process is described in considerable detail in U.S. Patent No. 4,800,159 and Canadian Patent No. 1,237,685 to Cetus Corporation, both of which are incorporated herein by reference.

The polymerase chain reaction is an efficient process for producing exponential quantities of a product relative to the number of reaction steps involved.

For the purposes of the present invention, the nucleotide sequence to be amplified includes the polymorphic restriction enzyme site under consideration and the nucleotide sequences flanking this site. This process is possible given that:

SUBSTITUTE SHEET

-14-

- (a) the ends of the restriction enzyme site or the flanking sequences are known in sufficient detail that oligonucleotides can be synthesized which are complementary to them and,
- (b) a small amount of the nucleotide sequence is available to initiate the chain reaction.

The product of the chain reaction will be a discrete nucleotide duplex with termini corresponding to the ends of the specific primers employed.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to the nucleotide sequence is induced, i.e. in the presence of nucleotides and an inducing agent and at a suitable temperature and pH. The primer is preferably single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The exact lengths of the primers may be different for each "template" or nucleotide region which is to be amplified. Generally, a balance must be struck with respect to the primer size. It must be large enough to be usefully specific to the template, that is, it must be homologous to a large enough region of the

SUBSTITUTE SHEET

-15-

templat so that other extraneous DNA (not related to the restriction enzyme site) with some degree of homology to the primer is not amplified to a significant extent. On the other hand, the size of the primer should not be so large as to be unyieldy and prohibitive in terms of cost. This balance may be achieved for at least some polymorphic enzyme restriction sites, with primers of between 10-50 nucleotides in length. The determination of the appropriate lengths of other primers is well within the skill of a technician in this area.

In a very preferred embodiment of the present invention, the primers are designed to flank asymmetrically the DNA sequence comprising the restriction enzyme site. The rationale behind this preferred embodiment is described further hereinbelow.

Accordingly, for each polymorphic restriction enzyme site under examination, two flanking primers are employed in the polymerase chain reaction. The "designer" primers permit the selective amplification of only the desired nucleotide sequences comprising the polymorphic restriction enzyme sites.

The DNA complement isolated from the cells of the individual is the "starting point" for the polymerase chain reaction. From this complement or mixture of nucleotide sequences, only the fraction comprising the polymorphic restriction enzyme site under consideration and for which the primers have been designed, will be amplified.

Accordingly, the greater the knowledge of the bases at both ends flanking the polymorphic

SUBSTITUTE SHEET

-16-

restriction enzyme site, the greater can be the specificity of the primers for the target site and thus the greater efficiency of the process.

The oligonucleotide primers may be prepared using any suitable method, such as, for example, the phosphotriester method (Narang, S. A. et. al., Methods of Enzymology, 68, 90 (1979)) and U.S. Patent No. 4,356,270) or the phosphodiester method (Brown, E. L. et. al., Methods of Enzymology, 68, 109 (1979)), or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage et. al., Tetrahedron Letters (1981), 22:1859-1862. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066. It is also possible to use a primer which has been isolated from a biological source (such as a restriction enzyme digest).

The specific nucleotide sequence is produced by using the nucleotide sequence from the sample isolated from the individual as a template. If the DNA is double-stranded, it is necessary to separate the strands before it can be used as a template, either as a separate step or simultaneously with the synthesis of the primer extension products. This strand separation can be accomplished by any suitable method including physical, chemical or enzymatic means. One physical method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely (greater than 99%) denatured. Typical heat denaturation may involve temperatures ranging from about 80 to 105° C for times ranging from about 1 to 10 minutes. Strand

SUBSTITUTE SHEET

-17-

separation may also be induced by an enzyme from the class of enzyme known as helicases or the enzyme RecA which has helicase activity and in the presence of RiboATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described by Kuhn Hoffmann-Berling, CSH-Quantitative Biology, 43:63 (1978), and techniques for using RecA are reviewed in C. Radding, Ann. Rev. Genetics, 16:405-37 (1982).

If the DNA isolated from the individual is single stranded, its complement is synthesized by adding one or two oligonucleotide primers thereto. If an appropriate single primer is added, a primer extension product is synthesized in the presence of the primer, an inducer or catalyst of the synthesis and the four nucleotides described below. The product will be partially complementary to the single-stranded nucleic acid and will hybridize with the nucleic acid strand to form a duplex of unequal length strands that may then be separated into single strands as described above to produce two single separated complementary strands. Alternatively and most preferably, two appropriate primers are added to the single-stranded nucleic acid and the reaction carried out.

After separation of the complementary strands (if in fact the sample comprises double-stranded DNA), the strands are ready to be used as templates for the synthesis of additional nucleic acid strands. This synthesis can be performed using any suitable method. Generally it occurs in an aqueous solution, preferably at physiological pH. Preferably, along with the inducing agent, a molar excess of the two

SUBSTITUTE SHEET

-18-

oligonucleotid primers is added to the solution containing the separated template strands. A large molar excess is preferred to improve the efficiency of the process.

The deoxyribonucleotides dATP, dCTP, dGTP and dTTP are also added to the synthesis mixture in adequate amounts, most preferably, from 50-200 μM of each, and the resulting solution is heated to about 90 - 100° C for several seconds to 10 minutes, preferably less than 1 minute. After this heating period, the solution is allowed to cool to between room temperature and 60°C, which is preferable for the primer hybridization or "annealing". The exact time necessary for annealing will depend on the guanidine and cytosine content and the length of the primers. To the cooled mixture is added an appropriate agent for inducing or catalyzing the primer extension reaction (herein called "inducing agent" or "enzyme catalyst"), and the reaction is allowed to occur under conditions known in the art. The synthesis may occur at from room temperature up to a temperature above which the inducing agent no longer functions efficiently. Thus, for example, if a heat stable DNA polymerase is used as inducing agent, the temperature may be as high as 85°C. It is to be understood that the higher the temperature for extension, the more specific the reaction becomes. The time period necessary for extension depends on the length of the template. Typically, about 1 minute per kilobase is sufficient. Most conveniently, the reaction occurs from room temperature to 72°C, depending on the enzyme.

Most preferably, the denaturing occurs at a temperature between 90-95°C, the annealing occurs at

SUBSTITUTE SHEET

-19-

a temperature between 40-60°C and the extension occurs at a temperature between 70-75°C.

Inducing agents suitable for use in the present invention may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase, and other enzymes, including heat-stable enzymes such as Taq DNA polymerase, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand.

The newly-synthesized strand and its complementary nucleic acid strand form a double-stranded molecule which is used in the succeeding steps of the polymerase chain reaction process. In the next step, the strands of the double-stranded molecule are separated using any of the procedures described above to provide single-stranded molecules.

New nucleic acid is synthesized on the single-stranded molecules. Additional inducing agent, nucleotides and primers may be added if necessary for the reaction to proceed under the conditions prescribed above. The synthesis will be initiated at one end of the oligonucleotide primers and will proceed along the single strands of the template to produce additional nucleic acid. After this step, half of the extension product will consist of the specific nucleotide sequence bounded by the two primers.

SUBSTITUTE SHEET

-20-

The steps of strand separation and extension product synthesis can be repeated as often as needed to produce the desired quantity of the specific nucleic acid sequence comprising the polymorphic restriction enzyme site. The amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion.

Optionally, a cofactor is added to the synthesis mixture to enhance the rate of synthesis. Suitable cofactors include, but are not limited to MgCl and KCl.

A cycle is one completion of the denaturing, annealing and extending steps. In a preferred form, a cycle may last from about 1 1/2-2 1/2 minutes. For the amplification of each restriction enzyme site, it is preferred that 25-35 cycles be completed, although it is to be understood that with an appropriate amount of template, fewer cycles may be necessary.

Optionally, each cycle may include, at its termination, an autoextension period. This is due to the fact that, in the initial extension period the primer may amplify more template than the DNA polymerase can handle, a condition referred to as a "substrate excess". The autoextension period effectively allows the DNA polymerase to work on the excess substrate.

The steps involved in the polymerase chain reaction procedure can be repeated independently subject only to continued addition of the appropriate primers, inducing agent and nucleotides. The amount

SUBSTITUTE SHEET

-21-

of original nucleic acid remains constant because it is not replicated, the amount of the sequence of interest comprising the polymorphic restriction enzyme site, hereinafter referred to as "amplified product" increases exponentially and will thus become predominant in the sample.

The general and preferred methods for carrying out the amplification are described in the U.S. Patent to Cetus described above. The technique is one in which those skilled in the art would be well-versed and confident and accordingly, exhaustive detail is not provided herein.

In one embodiment, two primers are added to the sample of nucleic acid. As described above, each of the primers is designed to complement the sequences flanking the polymorphic restriction enzyme site. In this embodiment only one desired region is amplified.

In order to expedite and economize the method of the present invention, a second embodiment is provided wherein primer sets for the regions flanking two or more polymorphic restriction enzyme sites may be added to the same reaction mixture for amplification, hereinafter referred to as "multiplex amplification".

In this embodiment, compatible primer sets are provided in one reaction mixture and the sequences amplified as outlined hereinabove. The mixture comprising the multiple products is then aliquoted and used in a second series of amplification steps, each one specific only to the nucleotide sequence comprising one particular

SUBSTITUTE SHEET

-22-

polymorphic restriction enzyme site. A primer set from the original pooled group is then added to one of the aliquots so that only one restriction enzyme locus is amplified in the second polymerase chain reaction. Hence, there is enough target DNA for analysis of all the restriction enzyme sites.

The amplified product comprising the sequences of nucleic acid including the polymorphic restriction enzyme site is then digested with the restriction enzyme corresponding to the site under consideration.

Conditions for the digestion step are recommended by the suppliers of the restriction enzymes. This information is readily available.

The digested samples of DNA are then separated by an appropriate separation procedure such as gel electrophoresis (agarose or polyacrylamide) or capillary electrophoresis. The presence or absence of a cleavage by the restriction enzyme is then identified, for example, by viewing the sample after separation by electrophoresis on an ethidium bromide - containing agarose gel. The DNA fragments are visualized when this electrophoretogram is placed over a light source such as an ultraviolet light box.

Each sample of DNA so prepared will, in general, be identified as either one, two or three bands on the gel.

Individuals who are homozygous for the absence of a particular restriction enzyme site, (-,-) will exhibit only one band corresponding to the distance between the two primers. This indicates the

SUBSTITUTE SHEET

-23-

absence of the site on both chromosomes. In contrast, individuals who are homozygous for the presence of a particular restriction enzyme site, (+,+) will exhibit two bands on the gel signifying the presence of the site on both chromosomes and this cleavage at the site by the enzyme. Lastly, individuals who are heterozygous for a particular restriction enzyme site, (+,-) will exhibit three bands on the gel indicating that the restriction enzyme site is present on only one of the two chromosomes.

The differentiation of the genotype of the individual based on one, two or three bands on an electrophoretic gel is due, in a large part, to the design of primers which flank the restriction enzyme site in an asymmetrical fashion. If, for example, an individual was homozygous for the presence of the restriction enzyme site and if the primers were designed to flank asymmetrically this site, two distinct bands would appear on the gel after enzyme digestion and separation. If, on the other hand, the primers had flanked symmetrically the site, only one band of double intensity would appear on the gel. Although it would still be possible to distinguish the homozygote (+,+) from the homozygote (-,-) based on the location of the bands, simple, elegant differentiation based on the variable number of bands would not be possible.

In order to determine the extent of variability at a particular restriction enzyme site, which will ultimately allow identification of the individual, genomic DNA samples, randomly selected from comparative members to which the individual belongs are assayed. Preferably, these comparative

SUBSTITUTE SHEET

-24-

members represent the general population. Each sample may be amplified as described herein to facilitate the detection of the restriction enzyme site. The nucleotide sequence comprising the polymorphic restriction enzyme site is subjected to the action of the restriction enzyme, and data on the presence or absence of the restriction enzyme site is recorded.

Using the process of the present invention, minimal population data is required for each restriction site or loci, as there are only two allelic types (the enzyme site is either present or absent). This is in sharp contrast to the other, currently employed detection techniques which rely on numerous size differentials between given alleles in a population.

In a preferred embodiment, the two-allele locus systems should have allelic frequencies in the general population to which the individual belongs of 30% to 70%, more preferably 40%-60% and most preferably close to 50% each. The closer the allelic frequencies are to 50%, the fewer restriction enzyme loci that will have to be tested in order to achieve highly discriminating results.

Appropriate human-specific polymorphic restriction enzyme sites include, but are not limited to, human adenine deaminase (HADA), prealbumin (PALB), adenine phosphoribosyl-transferase (APRT), and antithrombin III. In a preferred form of the present invention, the restriction enzyme site and the primers flanking this region are designed to be between 100 and 1,500 base pairs and most preferably less than 1,000 base pairs prior to enzyme digestion.

SUBSTITUTE SHEET

-25-

Appropriate restriction enzymes include, but are not limited to Taq I, Msp I, Fnu 4H1, Bam H1, Hind III, Pst 1, Eco R1, and Pvu II.

If an individual was tested at 16 loci (each loci has a 50% probability of the existence of a specific restriction enzyme site), and 8/16 were homozygous (for the presence or absence of the enzyme site), and 8/16 were heterozygous, the probability of finding another individual with the same combination of restriction enzyme sites (for these 16 loci) would be in the range of 1/17 million (refer to Example VI). This value is based on the assumption that all 16 of these loci are independent of one another i.e. the presence of a particular enzyme site at locus "A" does not influence the presence or absence of another enzyme site at locus "B". Forensic identification can be made by comparing the combination of restriction enzyme sites of the forensic sample of the individual with that of the suspect. In the event that the two patterns are identical, the allele (presence or absence of a specific enzyme site at a specific locus) frequencies are used to compute the likelihood of a chance match as described in Example VI hereinbelow.

Preferably, from 13 to 20 restriction enzyme loci are tested in order to determine the identity of an individual. More preferably, 15-20 loci may be tested, assuming that the presence of the polymorphic enzyme site varies between 30-70%. The closer that the presence of the restriction enzyme comes to 50%, the fewer loci must be tested.

SUBSTITUTE SHEET

-26-

The number of polymorphic restriction enzyme loci required for the method of the present invention should not be a deterrent, in fact, currently available technology such as the VNTR analysis also does demand a large number of population samples. There are, however, a number of time-saving measures that can be put into practice with the method of the present invention.

For example, primer sets which have compatible reaction temperatures may be put through the thermal cycler together (in individual tubes). Amplified products may all be loaded onto two gels. By following a very preferred embodiment of the present invention and thus ensuring that all amplified products are 1,000 base pairs or less, all loci can be electrophoresed through a polyacrylamide gel which is more sensitive than an agarose gel in this size range. If the casting and consistency of acrylamide gels becomes a time-consuming step, precasted gels are available from some suppliers. The advantage of using polyacrylamide gels is that vertical acrylamide gels lend themselves readily to automation as seen with DNA sequencing. Hence, in a laboratory equipped with a DNA extractor and an automatic thermal cycler, the manual input is reduced to loading the DNA extractor, aliquoting sample and polymerase chain reaction reactants, loading the polymerase chain reaction machine, setting up enzyme digest reactions and finally loading the gel. Thus, the time required for technical work is substantially reduced, resulting in an increase in the output of a laboratory and a reduction in the number of handling errors.

SUBSTITUTE SHEET

-27-

Although not absolutely essential, it is recommended that, for each polymorphic restriction enzyme site being tested, a control DNA sample of known length be digested and analyzed. The integrity of the restriction enzyme is important as the success of this identification process is dependent on its activity (digestion of the amplified DNA). Digesting a control sample of DNA with the restriction enzyme provides an internal check as to the reliability of the process.

Particularly in instances of human identification, when test results may be submitted before a court, it may be appropriate to sequence the primers to confirm the accuracy of the synthesis and/or it may be appropriate to hybridize the amplification products with a DNA probe for the particular region.

The main impetus behind the present invention, especially as it applies to forensic science, was to find a way to increase the quantity and to increase the quality of DNA available for analysis. With the method of the present invention, results can be obtained from severely degraded DNA as only the enzyme restriction site and a small portion of the flanking regions need to be intact. Multiplex amplification further increases the quantity and quality of a very small biological sample.

The method of the present invention is quick, efficient, cost-effective, non-radioactive (no exposure to radioisotopes) and reliable. The interpretation of the gel patterns is straightforward that an untrained person may

SUBSTITUTE SHEET

-28-

understand the results with minimal instruction. This simplicity is beneficial for court proceedings. The ability to amplify DNA allows for the analysis of DNA samples that are present in minute quantities. Hence, by analyzing small samples such as a few plucked hairs, it is possible to process criminal cases which have limited amounts of biological materials or paternity cases involving very young infants where the blood volume is restricted.

In addition, the results of the method described herein may be available within a few days as opposed to the time frame of four to six weeks necessary for the completion of current technology.

Lastly, the internal checks described herein allow the laboratory analyst to be confident that the interpretation of his/her results is correct.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

**EXAMPLE I: Isolation of Sample DNA and
 Amplification of APRT Locus**

Whole blood samples collected in EDTA vacutainer tubes were extracted for DNA according to the preferential cell lysis method (David Hoar-personal communication). Briefly, red blood cells were osmotically lysed with 5 volumes of a solution containing 0.017M of tris-HCl at pH 7.65 and 0.139M of NH_4Cl . The remaining intact nucleated cells were washed twice with saline before resuspension in two milliliters of HTE (100mM

SUBSTITUTE SHEET

-29-

tris-HCl, 40mM EDTA, pH 8.0). Two milliliters of lysis buffer (100mM tris-HCl, 40mM EDTA, 0.2% SDS, pH 8.0) were immediately injected into the nucleated cellular suspension to lyse the cells instantaneously. The released DNA was purified by phenol - chloroform extractions, with a final alcohol precipitation. The purified DNA was resuspended in LTE buffer (10mM tris-HCl, 1mM EDTA, pH 8.0) and their concentrations were determined with a DNA flurometer (Hoeffer).

Sequences for the 20-mer adenine phosphoribosyltransferase primers (APRT) were designed from published sequence data (T.P. Broderick, D.A. Schaff, A.M. Bertino, M.K. Dush, J.A. Tishfield and P.J. Stambrook, Comparative anatomy of the human APRT gene and enzyme: Nucleotide sequence divergence and conservation of a nonrandom CpG dinucleotide arrangement. Proc. Natl. Acad. Sci. USA, 84 (1987) 3349-3353). The two primers were designed such that the primer set flanked the polymorphic enzyme site. The variable sites for the APRT region is the Taq I site. The APRT primers were synthesized by the biochemistry department at The University of British Columbia.

The reaction mixture for the polymerase chain reaction (PCR) consisted of 0.3 μ M of each of the two primers, 1.7mM $MgCl_2$, 200 μ M of each of four dNTPs (Perkin Elmer Cetus/Pharmacia), 100ng genomic DNA, and 1.25 units Taq DNA polymerase (Perkin Elmer Cetus/Bethesda Research Laboratories), in a total volume of 50 μ l. Mineral oil was placed onto the surface of the mixture to prevent evaporation and/or condensation. A thermal cyclor (Perkin Elmer Cetus) was used to automate the PCR for 31 cycles. The temperatures were 94°C (30 secs) for denaturation, 61°C (30 secs) for annealing, 72°C (60

SUBSTITUTE SHEET

-30-

secs) for extension and with an autoextension f 3 seconds after each cycle.

**EXAMPLE II: Digestion of Amplified APRT Locus
 and Analysis of Results**

The aqueous fraction from Example I containing the amplified products was extracted from the top mineral oil layer. Five microliters were removed and applied to a 1.0% agarose (ethidium bromide) gel for electrophoretic separation to confirm the presence of a single band product by visual inspection over an ultraviolet light box (Spectroline Model TR-302). The remaining aqueous fraction was then precipitated with 2 volumes of 95% ethanol and 1/10 volume of 0.3M sodium acetate. The resulting pellet was resuspended in 12 ul sterile water and subsequently digested with the restriction enzyme. Conditions for the restriction enzyme digests are those recommended by the suppliers, Bethesda Research Laboratories (Taq I). Digested DNA samples were separated by electrophoresis on a 1.0% agarose (ethidium bromide) gel and viewed over an ultraviolet light box to allow for identification of PCR products.

In order to determine the extent of the variability at the Taq I site (APRT locus), genomic DNA samples, randomly selected from the population of the Greater Vancouver area, were assayed. Sample size for the APRT locus was 109 individuals. The amplified products all migrated as a single band for the locus (Fig. II), which is indicative of a presence of large quantities of fragments of one specific size. The visual absence of any other bands suggests that there was no significant amplification of other regions. Hind III digested lambda/phiX174 DNA were used as markers (Fig. II, lane m). The genomic DNA from 10 individuals was amplified and is

SUBSTITUTE SHEET

-31-

reflected in Fig. II lanes 1-10. The PCR product of the APRT primers was 763 base pairs (Fig. II). After digestion with Taq I, a minimum of one band and a maximum of three bands were visualized in an agarose (ethidium bromide) gel (Fig. III).

Individuals homozygous for the absence of the Taq I site at the APRT locus, have one band of 763 base pairs (Fig. III - lane 1). In contrast, homozygosity for the presence of the Taq I site resulted in two bands: 479 and 284 base pairs (Fig. III - lane 2). Heterozygotes for this enzyme site have all three bands: 763, 479 and 284 base pairs (Fig. III - lane 3).

Allele Frequencies:

The polymerase chain reaction was used to amplify a region (containing a polymorphic Taq I site) of the human APRT locus in 109 individuals. Fifty-eight (53.2%) individuals were homozygous for the presence of the Taq I site, 8 (7.3%) were homozygous for the absence of the site, and 43 (39.5%) were heterozygous. This indicated that the Taq I site is present at a frequency of 73% and its absence occurs at a frequency of 27%.

Restriction Enzyme Digest Control:

Since the success of this typing system is dependent upon the activity of the restriction enzyme digestion of the amplified DNA, a sample DNA preparation was included as a control for the digest reaction. A 422 base pair pBR322 fragment containing an internal centrally located Taq I site was constructed and used as the digestion control. This

SUBSTITUTE SHEET

-32-

422 base pair fragment (Fig. VI - lane 12) cuts into two fragments, differing only in ten base pairs. Hence, when electrophoresed through an agarose gel, the two fragments appear as one band at the 210 base pairs (Fig. VI - lane 13).

The control DNA fragment was added to each Taq I digest reaction of the APRT primer PCR products (Fig. VI). In each case, there was efficient digestion of both the PCR products and the control DNA (Fig. VI - lanes 1 to 11).

**EXAMPLE III: Isolation of Sample DNA and
Amplification of PALB Locus**

The DNA was isolated from the whole blood sample as indicated above in Example I.

Sequences for the prealbumin (PALB) primers were designed from published sequence data (H. Sasaki, N. Yoshioka, Y. Takage and Y. Sasaki, Structure of the Chromosomal Gene for Human Serum prealbumin. Gene, 37 (1985) 191-197). The two primers were designed such that the 'set' flanked the polymorphic enzyme site. The variable site for the PALB region is the Fnu 4HI site. The primers were synthesized by Pharmacia. Maxim and Gilbert sequencing was completed by Pharmacia on the PALB primers for sequence confirmation.

The polymerase chain reaction mixture consisted of 0.2uM of each of the two primers, 2.0mM $MgCl_2$, 200uM of each of the four dNTPs, 100ng genomic DNA, and 1.25 units Taq DNA polymerase, in a total volume of 50 ul. Mineral oil was placed onto the surface of the mixture to prevent evaporation and/or condensation. The reactions were carried out

SUBSTITUTE SHEET

-33-

as described in Example I for the APRT locus except that the PCR for the PALB was allowed to run for 30 cycles.

**EXAMPLE IV: Digestion of Amplified PALB Locus
 and Analysis of Results**

The aqueous fraction from Example III containing the amplified products was extracted from the top mineral oil layer. Five microliters were removed and applied to a 1.0% agarose (ethidium bromide) gel for electrophoretic separation to confirm the presence of a single band product by visual inspection over an ultraviolet light box (Spectroline Model TR-302). The remaining aqueous fraction was then precipitated with 2 volumes of 95% ethanol and 1/10 volume of 0.3M sodium acetate. The resulting pellet was resuspended in 12 μ l sterile water and subsequently digested with the restriction enzyme Fnu 4H1. Conditions for this restriction enzyme digest were those recommended by the suppliers, New England Biolabs (Fnu 4H1). Digested DNA samples were separated by electrophoresis on a 1.0% agarose (ethidium bromide) gel and viewed over an ultraviolet light box to allow for identification of PCR products.

In order to determine the extent of the variability at the Fnu 4H1 site (PALB locus), genomic DNA samples, randomly selected from the population of the Greater Vancouver area, were assayed. Sample size for the PALB locus was 109 individuals. The amplified products all migrated as a single band for the locus (Fig. IV), which is indicative of the presence of large quantities of fragments of one specific size. The visual absence of any other bands suggests that there were no significant amplification in other regions. The PCR product of the PALB

SUBSTITUTE SHEET

-34-

primers was 1011 base pairs (Fig. IV). After digestion with Fnu 4H1 (PALB), a minimum of one band and a maximum of three bands were visualized in an agarose (ethidium bromide) gel (Fig. V).

At the PALB locus, homozygosity for the absence of the Fnu 4H1 site resulted in only one band at 1011 base pairs (Fig. V - lane 4). An individual homozygous for the presence of the Fnu 4H1 site showed two bands; 654 and 357 base pairs (Fig. V - lane 2), while a heterozygote had three bands; 1011, 654 and 357 base pairs (Fig. V - lane 6).

The polymerase chain reaction was used to amplify a region (containing the polymorphic Fnu 4H1 site) of the human PALB locus in 109 individuals. Forty-seven (43.1%) people were homozygous for the presence of the Fnu 4H1 site, 8 (7.3%) were homozygous for the absence of the site, and 54 (49.5%) were heterozygous. This indicated that the Fnu 4H1 site is present at a frequency of 67% and its absence occurs at a frequency of 33%.

**EXAMPLE V: Multiplex (Simultaneous) Amplification
of the APRT and PALB Loci**

The DNA was isolated from the whole blood sample as indicated above in Example I.

The primer sets for both the APRT and PALB loci were prepared as indicated in Examples I and III, respectively.

Figure I is a representation of the amplified APRT and PALB loci. T and F represent the restriction endonuclease sites for Tag I and Fnu 4H1 respectively. Broken lines indicate the respective regions for primer binding. Thick arrows indicate

SUBSTITUTE SHEET

-35-

the direction of synthesis by Taq DNA polymerase. The sizes of the DNA fragments are indicated in base pairs.

Both of the primer sets, that is, two each for APRT and PALB were added to the same polymerase chain reaction mixture. Specifically, the reaction mixture consisted 0.2uM of the PALB primers and 0.3uM of the APRT primers, 1.7mM $MgCl_2$, 200uM of each of the four dNTPs (Perkin Elmer Cetus/Pharmacia), 100ng genomic DNA and 1.25 units Taq DNA polymerase in a total volume of 50ul. Mineral oil was placed onto the surface of the mixture to prevent evaporation and/or condensation. A thermal cycler was used to automate the reaction for 30 cycles. The temperature was 94°C (30 sec) for denaturation, 61°C (30 sec) for annealing, 72°C (60 sec) for extension and 3 seconds of autoextension after each cycle.

The aqueous fraction containing the amplified PCR products was extracted from the top mineral oil layer. Five microliters were removed and applied to a 1.0% agarose (ethidium bromide) gel for electrophoretic separation to confirm the presence of one band for each of the amplified loci, that is, one for the APRT locus and one for the PALB locus.

Indeed, two bands were observed after electrophoresis (Fig. VII - lanes 2 and 5). The top band corresponded to the 1011 base pair fragment at the PALB locus and the bottom band corresponded to the 763 base pair fragment at the APRT locus. Lanes 1 and 4 contain DNA amplified with only the APRT primers and lanes 3 and 6 contain DNA amplified with only the PALB primers.

SUBSTITUTE SHEET

Example VI: Statistical Analysis of 16 Loci

I. Assuming that the frequencies of alleles at each of the 16 loci were 0.7 (enzyme site present) and 0.3 (enzyme site absent) respectively.

According to Hardy Weinberg Equilibrium (H.W.E.)

where p^2 = frequency of homozygotes for the presence of the enzyme site (+/+)

$2pq$ = frequency of heterozygotes (+/-)

q^2 = frequency of homozygotes for the absence of the enzyme site (-/-)

$$\text{hence } (0.7)^2 + 2(0.7)(0.3) + (0.3)^2 = 1$$

$$0.49 + 0.42 + 0.09 = 1$$

therefore for 16 such loci, it is expected that an individual would be homozygous (+/+) in 8 (i.e. 0.49×16) loci, heterozygous (+/-) in 7 loci and homozygous (-/-) in 1 locus.

Probability of identity -

a) +/+ at those same 8 loci : $[(0.7)^2]^8 = [0.49]^8$

b) +/- at those same 7 loci : $[2(0.7)(0.3)]^7 = [0.42]^7$

SUBSTITUTE SHEET

-37-

$$c) \quad -/- \text{ at that same 1 locus : } [(0.3)^2]^1 = [0.09]^1$$

$$a \times b \times c = [0.49]^8 \times [0.42]^7 \times [0.9]^1$$

$$= 6.89 \times 10^{-7} = 1/1.4 \text{ million}$$

Accordingly, there would be only 1 person out of 1.4 million who would have that particular set or pattern at the 16 tested loci.

II. Assuming that the frequencies of alleles at each of the 16 loci were 0.5 (enzyme site present) and 0.5 (enzyme site absent).

$$\text{Then } p^2 = (0.5)^2 = 0.25$$

$$2pq = 2(0.5)(0.5) = 0.5$$

$$q^2 = (0.5)^2 = 0.25$$

Therefore in 16 such loci, it is expected an individual would be homozygous at 8 loci (i.e. $[0.25 + 0.25] \times 16$) and heterozygous at 8 loci.

Probability of identity -

$$a) \quad +/+ \text{ or } -/- \text{ at the same 8 loci : } [(0.5)^2]^8 = [0.25]^8$$

$$b) \quad +/- \text{ at the same 8 loci : } [2(0.5)(0.5)]^8 = [0.5]^8$$

$$a \times b = [0.25]^8 \times [0.5]^8$$

$$= 5.96 \times 10^{-8} = 1/16.8 \text{ million}$$

SUBSTITUTE SHEET

-38-

Accordingly, there would be only 1 person out of 16.8 million who would have that particular set or pattern at the 16 tested loci.

SUBSTITUTE SHEET

WHAT IS CLAIMED IS

1. A method for identifying an individual member of a species of organism which comprises analyzing the DNA of said individual in respect to the presence or absence in the genome of one or more polymorphic enzyme sites and based on comparisons of these results to the presence or absence of one or more of these polymorphic enzyme sites in the DNA of one or more comparative members of the species, so characterizing the individual member of the species.

2. The method according to Claim 1 wherein the presence or absence of one or more polymorphic restriction enzyme sites is determined by the following steps which comprise:

- (a) isolating DNA of individual to be analyzed;
- (b) amplifying at least one specific nucleotide sequence of said isolated DNA, which sequence comprises a polymorphic enzyme restriction site to produce amplified DNA;
- (c) digesting said amplified DNA with a restriction enzyme which is capable of cleaving the DNA at the polymorphic enzyme restriction site to produce DNA fragments;
- (d) separating the digested DNA fragments; and
- (e) identifying the presence or absence of cleavage at the restriction enzyme site.

3. The method according to one of Claims 1 or 2 wherein said comparative members of the species are

SUBSTITUTE SHEET

r representative of the general population to which the individual member belongs.

4. The method according to one of Claims 1 or 2 wherein there is one comparative member which is the same as the individual member.

5. The method according to one of Claims 1 or 2 wherein the comparative members are selected from the group comprising the mother of the individual member, the father of the individual member and a sibling of the individual member.

6. The method according to one of Claims 1 or 2 wherein said species to which the individual belongs is selected from the group comprising humans, animals, plants, viruses, bacteria, algae and fungi.

7. The method according to one of Claims 1 or 2 wherein the DNA is obtained from cells of adult, juvenile, fetal or embryonic tissue.

8. The method according to Claim 2 wherein the DNA isolated from the individual member is single stranded and the specific nucleotide sequence which comprises a polymorphic enzyme restriction site is amplified by the steps comprising:

(a) providing two primer regions of nucleotides, each complementary with one of the flanking ends of the nucleotide sequence of the polymorphic restriction enzyme site;

(b) allowing reaction between said primer regions and said single stranded DNA under

SUBSTITUTE SHEET

-41-

conditions such that the primer directs the synthesis of an extension product which is complementary to a single strand of the restriction enzyme site, said extension product then being a template for the synthesis of the extension product of the other primer.

9. The method according to Claim 8 wherein the DNA is amplified using an inducing agent and four different nucleotides.

10. The method according to Claim 8 which is repeated at least once.

11. The method according to Claim 9 wherein the inducing agent is selected from the group comprising E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, other available DNA polymerases such as Taq DNA polymerase, reverse transcriptase and other enzymes including heat-stable enzymes.

12. The method according to Claim 8 wherein the two primers are designed to flank asymmetrically the polymorphic restriction enzyme site.

13. The process according to Claim 9 wherein each of the primers comprise a sequence of between 10 and 50 nucleotides.

14. The method according to Claim 8 wherein each of the two primers and their respective polymorphic restriction enzyme site are between 100 and 1,500 nucleotides in length.

SUBSTITUTE SHEET

15. The method according to Claim 14 wherein each of the two primers and their polymorphic restriction enzyme site are a maximum of 1,000 nucleotides in length.
16. The method according to one of Claims 1 or 2 wherein said restriction enzyme is selected from the group comprising Taq I, Msp I, Fnu 4H1, Bam H1, Hind III, Pst I, Eco R1 and Pvu II.
17. The method according to Claim 2 wherein said digested DNA is separated in step (d) by gel electrophoresis.
18. The method according to Claim 1 wherein said polymorphic restriction enzyme site is present in one or more comparative members of the species at a frequency of between 30% and 70%, more preferably between 40% and 60%.
19. The method according to Claim 18 wherein the polymorphic restriction enzyme site is present at a frequency of approximately 50%.
20. The method according to one of Claims 1 or 2 wherein between 13 and 20 polymorphic restriction enzyme sites are analyzed.
21. A method for identifying an individual member of a species of organism which comprises analyzing the DNA of said individual member in respect to the presence or absence in the genome of one or more polymorphic restriction enzyme sites and

SUBSTITUTE SHEET

-43-

based on comparisons of these results to the presence or absence of one or more of these polymorphic restriction enzyme sites in the DNA of a forensic sample and in the DNA of the general population to which the individual member belongs, so determining whether the forensic sample originates from the individual member.

22. The method according to Claim 21 wherein the presence or absence of one or more polymorphic restriction enzyme sites is determined by the following steps which comprise:

- (a) isolating DNA of the individual member to be analyzed;
- (b) amplifying at least one specific nucleotide sequence of said isolated DNA, which sequence comprises a polymorphic enzyme restriction site to produce amplified DNA;
- (c) digesting said amplified DNA with a restriction enzyme which is capable of cleaving the DNA at the polymorphic enzyme restriction site to produce DNA fragments;
- (d) separating the digested DNA fragments; and
- (e) identifying the presence or absence of cleavage at the restriction enzyme site.

23. The method according to Claim 21 wherein the forensic sample is isolated from the scene of a crime.

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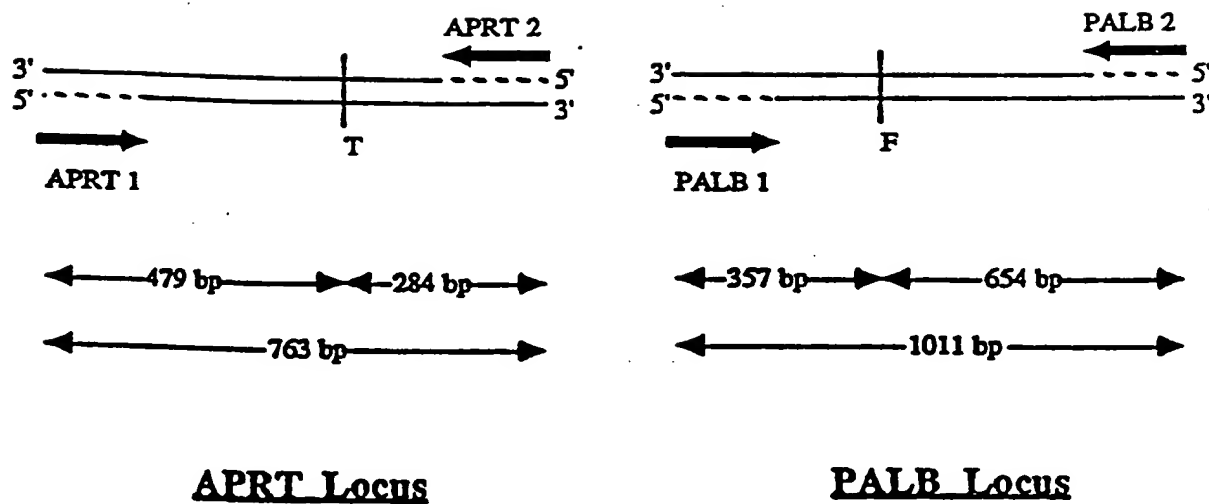
-44-

24. The method according to one of Claims 21 or 22 wherein said forensic sample comprises cells isolated from blood, tissue, semen, urine, or other body fluids.

SUBSTITUTE SHEET

1/7

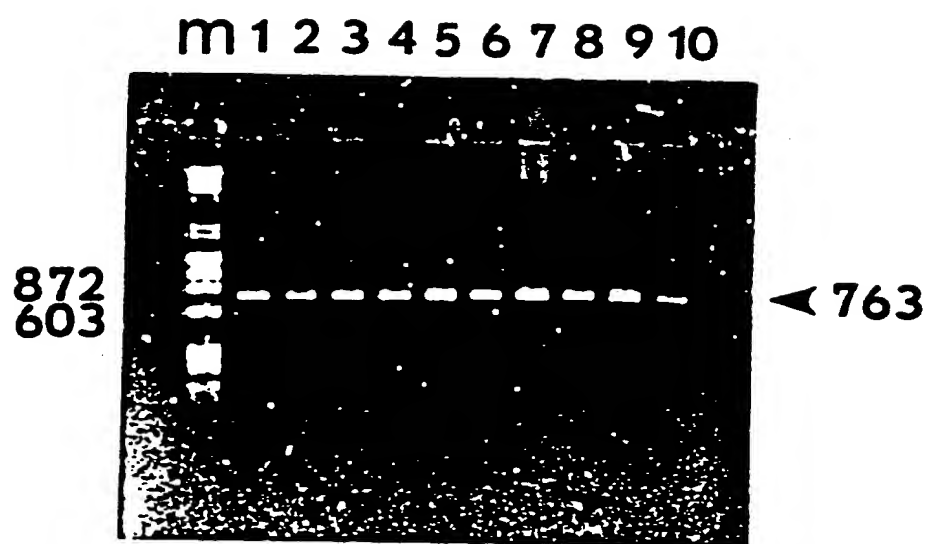
FIGURE 1



SUBSTITUTE SHEET

2/7

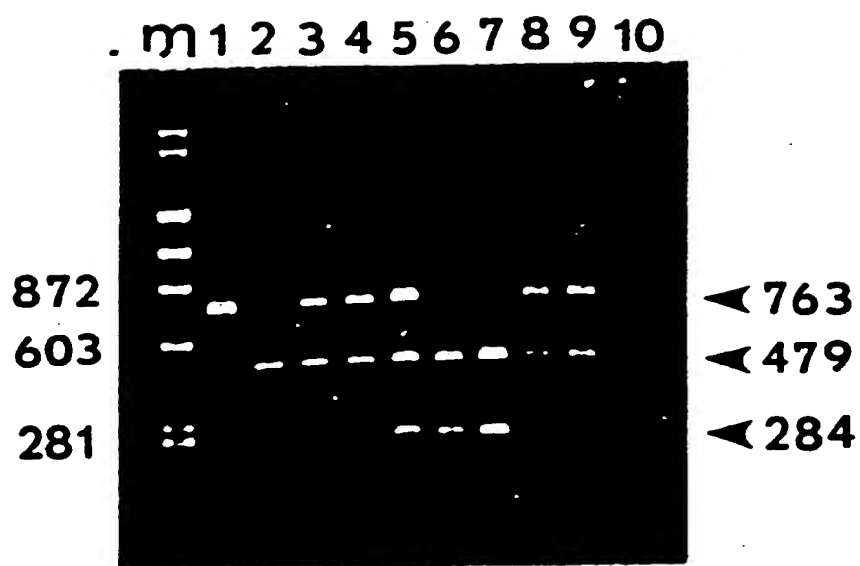
FIGURE 2



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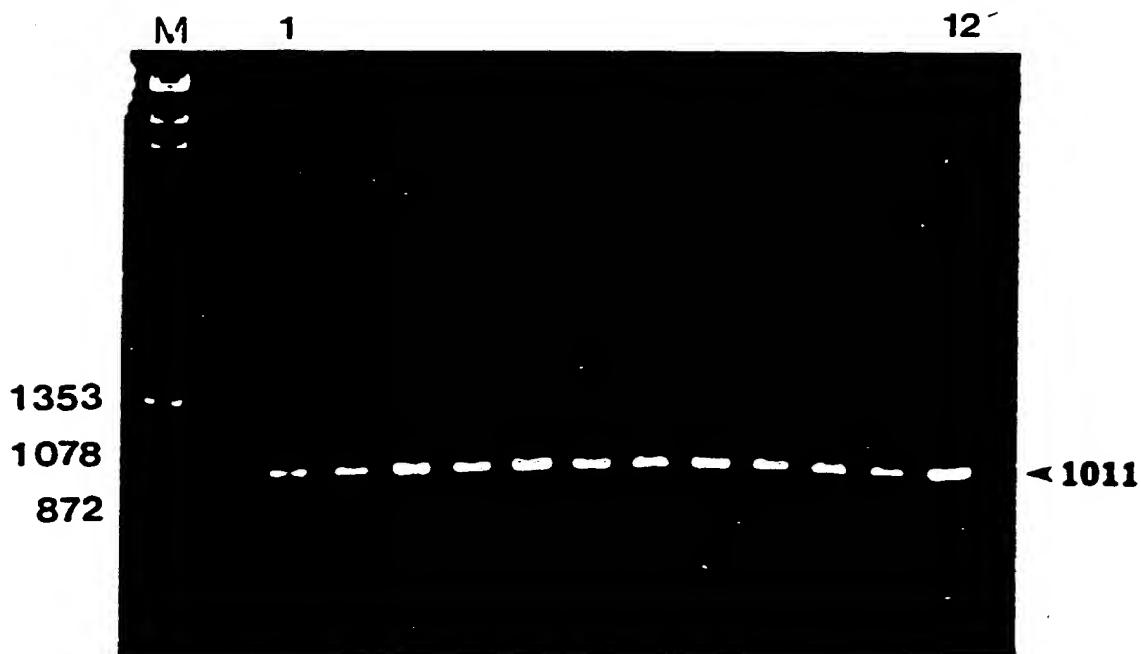
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FIGURE 3



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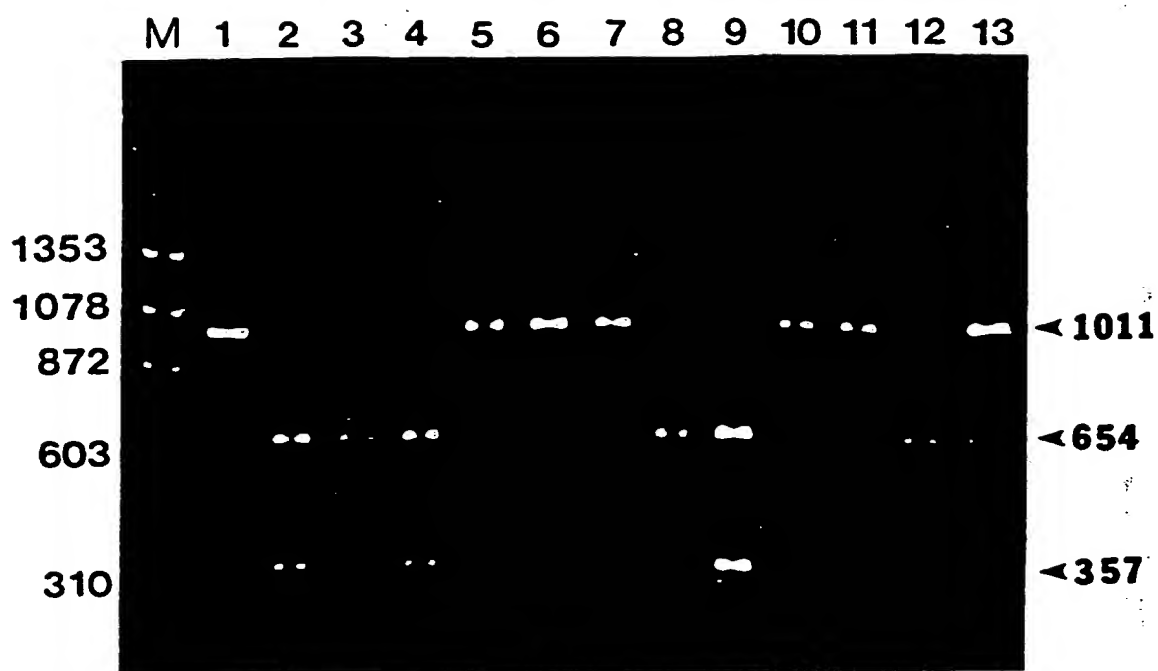
FIGURE 4



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5/7

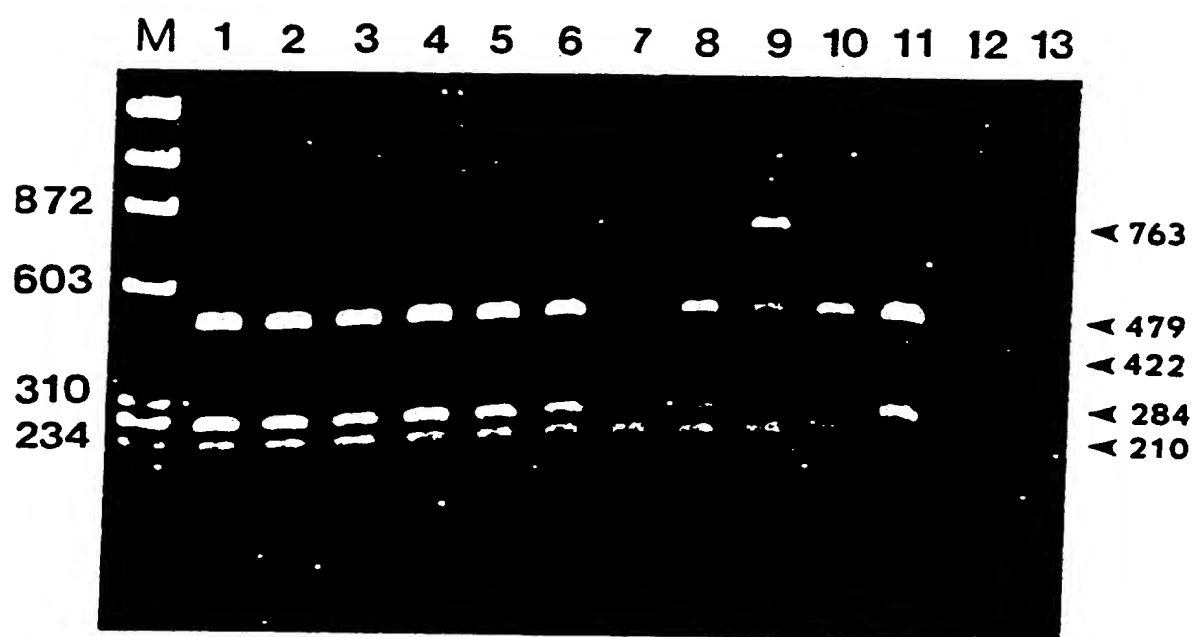
FIGURE 5



SUBSTITUTE SHEET

6/7

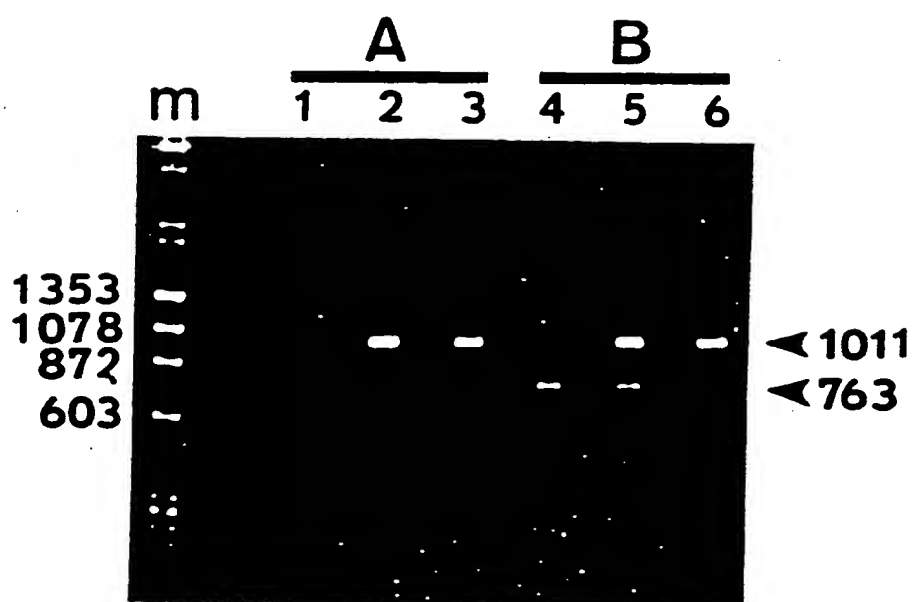
FIGURE 6



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7/7

FIGURE 7



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 91/00141

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁵: C 12 Q 1/68 // C 12 P 19/34

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System

Classification Symbols

IPC⁵

C 12 Q

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰

Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²

Relevant to Claim No. ¹³

X	WO, A, 8602101 (BIOTECHNICA LTD) 10 April 1986 see the whole document --	1,4-6,16,17 20
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X	EP, A, 0076123 (WEBSTER, J.) 6 April 1983 see abstract; page 14, line 25 --	1,3,6,7,16, 17,20 2,8-11,13,22
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Y	--	2,8-11,13,22
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Y	EP, A, 0200362 (CETUS CORP.) 10 December 1986 see example 5, column 51, lines 27-50 --	2,8-11,13,22
---	---	--------------

X	GB, A, 2135774 (ACTAGEN INC.) 5 September 1984 see the whole document cited in the application (as family member CA 1215304) --	1,3-7,16-21 ./.
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¹⁴ Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another claim or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

15th July 1991

Date of Mailing of this International Search Report

23. 08. 91

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

Natalie Weinberg

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	EP, A, 0388053 (CHEMBIOMED LTD) 19 September 1990 see claim 1 -----	1,3,6,17

Form PCT/ISA 210(extra sheet) (January 1985)

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

CA 9100141
SA 46921

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 07/08/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		GB-A, B 2189598	28-10-87
		JP-T- 62500423	26-02-87
EP-A- 0076123	06-04-83	US-A- 4717653	05-01-88
		AU-A- 6473890	17-01-91
		AU-B- 606873	21-02-91
		AU-A- 7477787	08-10-87
		AU-B- 562545	11-06-87
		AU-A- 8993182	08-04-83
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		DE-A- 3279284	19-01-89
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		AU-B- 586233	06-07-89
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		AU-B- 591104	30-11-89
		AU-A- 5532386	02-10-86
		CA-A- 1237685	07-06-88
		EP-A- 0201184	12-11-86
		JP-A- 62000281	06-01-87
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